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(71) Applicant: BAYLOR COLLEGE OF MEDICINE
One Baylor Plaza
Houston, TX 77030(US)

(72) Inventor: Caskey, Charles T.
6402 Belmont Street
Houston Texas 77005(US)
Inventor: Chamberlain, Jeffrey S.
3117 Cason
Houston Texas 77035(US)
Inventor: Gibbs, Richard A.L.
3602 Gramercy
Houston Texas 77025(US)
Inventor: Rainer, Joel, E.
2100 Commonwealth R.
Houston Texas 77006(US)
Inventor: Nguyen, Phi Nga
2300 Old Spanish Trail, Apt. 1032
Houston Texas 77054(US)

(74) Representative: Adams, William Gordon et al
RAWORTH, MOSS & COOK 36 Sydenham Road
Croydon Surrey CR0 2EF(GB)

(54) Multiplex genomic DNA amplification for deletion detection.

(57) The present invention relates to a method for detecting multiple DNA sequences simultaneously. The method involves amplification of multiple sequences simultaneously by annealing a plurality of paired oligonucleotide primers to single stranded DNA. One member of each pair is complimentary to the sense strand of a sequences and the other member is complimentary to a different segment of the anti-sense strand of the same sequence. The amplification occurs by alternately annealing and extending the primers. The invention also includes oligonucleotide primer sequences helpful in detecting genetic diseases and/or exogenous DNA sequences.

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Multiplex Genomic DNA Amplification for Deletion Detection

Field of the Invention

This invention relates to the field of simultaneous detection of deletions in genomic DNA sequences by the process of amplification of multiple sequences within the hemizygous or homozygous genome. The nucleic acid sequences are amplified by the process of simultaneous multiple repetitive reactions. This method of deletion detection is useful in a variety of areas including screening for genetic disease, and animal husbandry. Multiplex DNA amplification is also applicable to the simultaneous analysis of multiple genomic sequences and is useful in forensic medicine, disease screening, and in the development of recombinant or transgenic organisms.

Background

This invention is an improvement on currently established procedures for the detection of genetic diseases resulting from mutations and deletions in genomic DNA sequences. Prenatal diagnosis and carrier detection of many X-linked diseases is available via Southern analysis using full length CDNA clones. Unfortunately, there are several major limitations that prevent widespread and routine use of Southern analysis for diagnosis of genetic disease. In many of the X-linked diseases, the defective sequences are unknown and probes are unavailable. In other diseases, such as X-linked muscular dystrophy, there are multiple exons, at least 60, scattered over a large area of genomic DNA, approximately 2.4 million bases. The introns average 35 Kb in length. In the case of muscular dystrophy, at least 7-9 separate cDNA subclones are necessary for Southern blot analysis to resolve each exon-containing restriction fragment for hyplotype assignment or diagnosis of genomic alterations. Furthermore, Southern analysis is an expensive, tedious, and time-consuming technique that requires the use of radioisotopes, making it unsuitable for routine use in clinical laboratories.

An alternative to Southern analysis for mutation and deletion detection is the polymerase chain reaction (PCR) described by Mullis et al. in U. 5. Patent No. 4,683,195 which issued on July 28, 1987 and by Mullis in U. S. Patent No. 4,683,202 which issued on July 28, 1987. With PCR, specific regions of a gene can be amplified up to a million-fold from nanogram quantities of genomic DNA. After amplification the nucleic acid sequences can be analyzed for the presence of mutant alleles either by direct DNA sequencing or by hybridization with allele-specific oligonucleotide probes. The PCR technique has proven useful in the diagnosis of several diseases including β-thalassemia, hemophilia A, sickle cell anemia and phenylketonuria. Routine screening for genetic diseases and exogenous DNA sequences, such as virus, with PCR, has been limited by the ability to conduct tests for only a single sequence at a time. Screening for a plurality of possible DNA sequences requires a cumbersomely large number of separate assays, thus increasing the time, expense, and tedium of performing such assays. For example, in some diseases, such as Duchenne muscular dystrophy (DMD), PCR diagnosis has been limited since point mutations leading to DMD have not been identified. Approximately 60% of the cases of DMD are due to deletions. The other 40% are unknown at present, but probably involve mutations of the intron-exon splice sites or the creation of premature stop codons. Thus a large gene like the DMD gene must be screened with multiple assays.

In both U. S. Patent Nos. 4,683,195 and 4,683,202, procedures are described for amplification of specific sequences. Both patents describe procedures for detecting the presence or absence of at least one specific nucleic acid sequence in a sample containing a mixture of sequences. Although the patents claim at least one sequence and state that multiple sequences can be detected, they do not provide an effective procedure for amplifying multiple sequences at the same time. In the examples, single sequences are amplified or multiple sequences are amplified sequentially. Adding primers for a second sequence is usually possible, but when primers for more than two sequences are added the procedure falls apart. The present application is an improvement on the PCR method and solves the problems encountered when primers for multiple sequences are reacted simultaneously. The present invention describes a procedure for simultaneous amplification of multiple sequences, and the application of this multiplex amplification procedure to detect a plurality of deletions within the same gene or within multiple genes.

The procedures of the present application provide improved methods for the detection of deletions in hemizygous genes on the X and Y chromosomes. The procedures are effective in detecting genetic

diseases caused by deletions on the X or Y chromosome, for example, DMD. They are also effective in detecting homozyous deletions and may be used to simultaneously screen for many possible homozygous or hemizygous deletions as long as parts of the appropriate genetic sequences are known. The procedure for multiplex amplification also enables simultaneous analysis of multiple genetic loci regardless of the presence or absence of deletions.

Summary of the Invention

An object of the present invention is a method for simultaneously detecting deletions at a plurality of genomic DNA sequences.

An additional object of the present invention is to detect X-linked genetic diseases.

A further object of the present invention is the diagnosis of DMD.

A further object of the present invention is to simultaneously analyze multiple genetic loci for polymorphisms and/or non-deletion mutations.

Thus, in accomplishing the foregoing objects there is provided in accordance with one aspect of the present invention, a method for simultaneously detecting deletions at a plurality of genomic DNA sequences, comprising the steps of:

Treating said genomic DNA to form single stranded complementary strands;

Adding a plurality of paired oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand;

Annealing the plurality of primers to their complementary sequences;

Simultaneously extending said plurality of annealed primers from each primer's 3′ terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separating from their complement, serving as templates for the synthesis of an extension product from the other primer of each pair;

Separating said extension products from said templates to produce single-stranded molecules;

Amplifying said single stranded molecules by repeating at least once, said annealing, extending and separating steps; and

Identifying said amplified extension products from each different sequence.

Additional embodiments include detection of deletions at a plurality of genomic DNA sequences on the X and Y chromosomes or on autosomal chromosomes when the deletions are homozygous. A variety of X-linked diseases can be detected including ornithine transcarbamylase deficiency, hypoxanthine phosphoribosyltransferase deficiency, steroid sulfatase deficiency and X-linked muscular dystrophy.

In another embodiment, X-linked muscular dystrophy is detected using a plurality of paired primers which are complementary to different sequences within the gene coding for the protein dystrophin. Other embodiments include multiple oligonucleotide primers useful in detecting X-linked genetic disease.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure when taken in conjunction with the accompanying drawings.

Brief Discussion of the Drawings

The invention will be more readily understood from a reading of the following specification and by references to the accompanying drawings, forming a part thereof:

Figure 1 is a schematic representation of the DMD gene illustrating the approximate size of the locus, the position of the amplified fragments and the location of the genomic regions that have been cloned and sequenced.

Figure 2 is an example of a PCR reaction used to detect a deletion in fetal DNA for prenatal diagnosis.

Figure 3 represents the multiplex DNA amplification of lymphoblast DNA from unrelated male DMD patients. A. and B. show two sets of ten samples. Each DRL # refers to the R.J. Kleberg Center for Human Genetics Diagnostic Research Laboratory family number. MW: Hae III digested ϕX174 DNA. (-): no template

DNA added to the reaction. The relationship between the amplified region and the region on the gene is indicated to the right of A. The letters correspond to those on Figure 1.

Figure 4 represents Multiplex DNA amplification for prenatal diagnosis of DMD. Shown are the results of amplification using DNA from an affected male (AM; lymphoblast DNA) and a male fetus (MF; cultured amniotic fluid cell DNA) from six different families. Both the affected male and the fetal DNAs of DRL #s 521 and 531 display a deletion of region f (Fig. 1) diagnosing these fetuses as affected. In DRL # 43C the affected male is deleted for all regions except f, while the fetus is unaffected. The affected male in DRL # 483 is deleted for region a, while the male fetus is unaffected. Neither of the samples from DRL #s 485 or 469 displayed a deletion with this technique.

Figure 5 represents Multiplex DNA amplification from chorionic villus specimen (CVS) DNA. Both the affected male (AM; lymphoblast DNA) and the male fetus (MF; CVS DNA) from DRL # 92 display a deletion of regions e and f (Fig. 1), diagnosing the fetus as affected. CVS DNA from DRL # 120 did not display a deletion with this technique.

Figure 6 shows amplification of seven exon regions of the DMD locus.

The drawings are not necessarily to scale and certain features of the invention may be exaggerated in scale or shown in schematic form in the interests of clarity and conciseness.

Detailed Description

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein, without departing from the scope and spirit of the invention.

The term "oligonucleotide primers" as used herein defines a molecule comprised of more than three deoxyribonucleotides or ribonucleotides. Its exact length will depend on many factors relating to the ultimate function and use of the oligonucleotide primer, including temperature, source of the primer and use of the method. The oligonucleotide primer can occur naturally, as in a purified restriction digest, or be produced synthetically. The oligonucleotide primer is capable of acting as an initiation point for synthesis when placed under conditions which induce synthesis of a primer extension product complementary to a nucleic acid strand. The conditions can include the presence of nucleotides and an inducing agent such as a DNA polymerase at a suitable temperature and pH. In the preferred embodiment, the primer is a single-stranded oligodeoxyribonucleotide of sufficient length to prime the synthesis of an extension product from a specific sequence in the presence of an inducing agent. In the deletion detection procedure, the oligonucleotides are usually at least greater than 12 mers in length. In the preferred embodiment, the oligonucleotide primers are about 18 to 29 mers in length. Sensitivity and specificity of the oligonucleotide primers are determined by the primer length and uniqueness of sequence within a given sample of template DNA. Primers which are too short, for example, less than about 12 mers may show non-specific binding to a wide variety of sequences in the genomic DNA and thus are not very helpful. In the preferred embodiment, the oligonucleotide primer is usually selected for its ability to anneal to intron sequences in the proximity of the 5′ or 3′ end of the exon or to anneal to a sequence at the intron-exon junction. Since the known deletion defects resulting in genetic diseases result from deletions that include the exons or intron-splice site regions, it is preferable to have primers complementary to intron sequences.

Each primer pair herein was selected to be substantially complementary to the different strands of each specific sequence to be amplified. Thus, one primer of each pair is sufficiently complementary to hybridize with a part of the sequence in the sense strand and the other primer of each pair is sufficiently complementary to hybridize with a different part of the same sequence in the anti-sense strand. Thus, although the primer sequence need not reflect the exact sequence of the template, the more closely it does reflect the exact sequence the better the binding during the annealing stage.

Within a primer pair, each primer preferably binds at a site on the sequence of interest distant from the other primer. In the preferred embodiment the distance between the primers should be sufficient to allow the synthesis of an extension product between the two binding sites, yet close enough so that the extension product of each primer, when separated from its template, can serve as a template for the other primer. The extension products from the two paired primers are complementary to each other and can serve as templates for further synthesis. The further apart the binding sites, the more genomic DNA which can be screened. However, if the distance is too great the extension products will not efficiently overlap with the primers and thus amplification will not occur.

As used herein the term "extension product" refers to the nucleotide sequence which is synthesized from the 3′ end of the oligonucleotide primer and which is complementary to the strand to which the

oligonucleotide primer is bound.

As used herein the term "differentially labeled" shall indicate that each extension product can be distinguished from all the others because it has a different label attached or is of a different size or binds to a specifically labelled oligonucleotide. One skilled in the art will recognize that a variety of labels are available. For example, these can include radioisotopes, fluorescers, chemiluminescers, enzymes and antibodies. Various factors affect the choice of the label. These include the effect of the label on the rate of hybridization and binding of the primer to the DNA, the sensitivity of the label, the ease of making the labeled primer, probe or extension products, the ability to automate, available instrumentation, convenience and the like. For example, a different radioisotope could be used such as ^{32}P, ^{3}H, or ^{14}C; a different fluorescer such as fluorescin, tetramethylrhodamine, Texas Red or 4-chloro-7- nitrobenzo-2-oxa-1-diazole (NBD); or a mixture of different labels such as radioisotopes, fluorescers and chemiluminescers. Alternatively, the primers can be selected such that the amplified extension products for each sequence are of different lengths and thus can be separated by a variety of methods known in the art. Similarily, the extension products could include a restriction fragment length polymorphism which could be used to distinguish different extension products. In these examples, each primer or its extension product can be differentiated from all the other primers when they are in a mixture. Alternatively, probes which bind to the amplified extension products could be labeled and sets of probes which distinguish alleles of a single sequence within a multiplex DNA amplification reaction may be used whether or not labelled.

Each specific, different DNA sequence, which is to be detected herein, can derive from genomic DNA of the organism or exogenous DNA such as virus, bacteria or parasites. The source of genomic DNA from the organism to be tested can be blood, hair or tissue (including chorionic villi, amniotic cells, fibroblasts and biopsies). The source of DNA may be freshly obtained or have been suitably stored for extended periods of time. The DNA must be of sufficient quality to permit amplification. The genomic DNA can be prepared by a variety of techniques known to one skilled in the art.

As used herein, the term "deletion" refers to those genomic DNA sequences in which one or more nucleic acid base has been deleted from the sequence and thus is no longer present in the gene. The size of the deletion can affect the sensitivity of the amplification procedure. Generally, the larger the deletion the larger the sensitivity.

Any specific known nucleic acid sequence can be detected by the present method. Preferably, at least part of the sequence is deleted from the genome. It is only necessary that a sufficient number of bases at both ends of the sequence be known in sufficient detail to prepare oligonucleotide primers which will hybridize to the different strands of the desired sequence at relative positions along the sequence.

The oligonucleotide primers may be prepared using any suitable method, for example, phosphyltriester and phosphyldiester methods or automated embodiments thereof, the synthesis of oligonucleotides on a modified solid support, the isolation from a biological source (restriction endonuclease digestion), and the generation by enzymatically directed copying of a DNA or RNA template.

One embodiment of the present invention is a method for simultaneously detecting deletions at a plurality of DNA sequences, comprising the steps of: treating said DNA to form single stranded complimentary strands; adding a plurality of paired oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complimentary to a part of the sequence in the sense-strand and the other primer of each pair substantially complimentary to a different part of the same sequence in the complimentary anti-sense strand; annealing the plurality of primers to their complimentary sequences; simultaneously extending said plurality of annealed primers from each primer's 3′ terminus to synthesize an extension product complimentary to the strands annealed to each primer, said extension products, after separation from the complement, serving as templates for the synthesis of an extension product from the other primer of each pair; separating said extension products from said templates to produce single-stranded molecules; amplifying said single-stranded molecules by repeating, at least once, said annealing, extending and separating steps; and identifying said amplified extension product from each different sequence.

One preferred embodiment of the present invention is a method for detecting deletions at a plurality of genomic DNA sequences, wherein said sequences are selected from a group of sequences on the X and Y chromosomes. It is preferrable to detect hemizygous genes on the X and Y chromosomes, since this increases the level of sensitivity. When the procedure is used to detect the heterozygous state, it requires quantitative measurement, and thus is much less efficient than detecting the presence or absence of sequences as is done for hemizygous genes. For example, if part of an exon has been deleted the multiplex amplification method of the present invention will detect this by either failing to produce an oligonucleotide sequence or by production of an oligonucleotide sequence of a different size. Furthermore multiple exons can be screened at the same time. Thus, it is easy to detect the presence of a deletion.

However, in looking at heterozygous states, where the chromosomes have one normal gene and one deleted gene, the normal gene will produce a normal product, and thus there is the necessity to measure the quantitative difference in the production of extension products.

A second embodiment of the present invention is to permit simultaneous amplification of multiple, possibly unrelated sequences for the purpose of their simulataneous analysis. Such analysis may simply involve the determination of whether exogenous sequences (virus, bacteria or other parasites) are present within a sample of DNA, or might involve the detection of polymorphisms or mutations within a plurality of sequences. The polymorphisms or mutations can be detected by a variety of methods well known to those skilled in the art. The methods include, but are not limited to, direct DNA sequencing, allele-specific oligonucleotide hybridization, and competitive oligonucleotide priming.

The multiplex genomic DNA amplication method is preferably used to detect X-linked diseases resulting from deletions in the genomic DNA sequence. Genetic diseases can be caused by a variety of mechanisms including mutations and deletions. The procedure described herein was developed for detection of genetic diseases which result from deletions within the genome. Examples of some X-linked diseases which are candidates for the use of multiplex genomic DNA amplification are ornithine transcarbamylase deficiency, hypoxanthine phosphoribosyltransferase deficiency, steroid sulfatase deficiency and X-linked muscular dystrophy. Other disorders on the X chromosome or genes on the Y chromosome can also be easily detected. The procedure is also applicable to the detection of any set of known point mutations within a set of genomic sequences. The procedure is also applicable to the simultaneous detection of any set of exogenous DNA sequences in a given DNA sample. The procedure is also applicable to the simultaneous detection of any set of polymorphic or variable tandemly repetitive sequences within a genone.

The advantages of the multiplex amplification system are that numerous diseases or specific DNA sequence alterations can be detected in the same assay. For example, primers to hypoxanthine phosphoribosyltransferase deficiency, steroid sulfatase deficiency, X-linked muscular dystrophy, ornithine transcarbamylase deficiency and other X-linked diseases can all be run simultaneously on the same sample. Furthermore, the multiplex amplification procedure is useful for very large genes with multiple exons, such as the dystrophin gene. Because of the large size of the dystrophin locus, Mullis type PCR amplification is not able to scan the whole gene in one assay. Thus, it is necessary for multiple site amplification within the gene to detect all possible deletions which could result in disease. Deletions at the DMD locus can encompass any of the approximately 60 plus exons which are distributed over more than 2 million bases of DNA. Virtually all of these exons are separated by large introns and so up to 60 separate reactions could be required for complete analysis of DMD deletions. To simplify this task, the present invention of a multiplex genomic DNA amplification for deletion detection can be employed to perform simultaneous examination of multiple exons. For example, oligonucleotide primers flanking separate DMD gene exons can be synthesized and combined and used for multiplex DNA applications. At present, up to at least 7 different DMD gene sequences have been examined simultaneously. The entire procedure for the multiplex amplification from start-up to photography of the results takes less than 5 hours. The relative locations of the amplified regions do not affect the results and exons have been amplified which have been separated by at least 1000 kb. The PCR amplification technique of Mullis is adequate for one and possibly two pair of primers, but when greater than two pairs of primers are used the procedure will not adequately amplify all the appropriate sequences.

One skilled in the art readily appreciates that as more exon gene sequences become available the applicability of this test will expand to examine for deletions in multiple genes at the same time or examine multiple sites within the same gene at the same time. The later example is important for genes such as dystrophin which are so large that primers annealed to the ends of the gene will not traverse the whole gene sequence. Thus the necessity of doing multiple analysis to detect deletions in different regions of the gene. In addition, as specific mutations within multiple unrelated genes become known, multiplex DNA amplification can be applied to simultaneously assay for the presence of any of these mutations.

Furthermore, as specific or highly variable DNA sequence polymorphisms become known in various genetic Locl, multiplex DNA amplification can be used to simultaneously analyze these polymorphisms to determine the haplotype or to determine the identity or source of DNA (genetic footprinting).

The number of analyses which can be run simultaneously is unlimited, however, the upper limit is probably about 20 and is dependent on the size differences required for resolution and/or the number of labels or methods which are available to resolve the extension products. The ability to simultaneously amplify only 9 exons.would allow the detection of greater than 90% of all known DMD deletions in a single reaction. The ability to simultaneously amplify even as few as 10 exons allows the rapid and simple diagnosis of DMD deletions using only a few separate reactions. Assuming that there are about 60 exons in the DMD gene and that the exons are widely separated such that primers are needed for every exon, a

maximum of 6 separate assays is needed to detect all deletions in this gene. Under the same assumptions the Mullis PCR method would require 60 separate reactions to detect the deletions in this gene. Thus, as the size of the gene increases and the number of exons which cannot be detected together increases the advantages of this method are greatly enhanced. Furthermore, use of an automatic PCR apparatus (such as that produced by Perkin-Elmer/Cetus) and DNA sequencing machines will facilitate resolution and detection of amplified DNA fragments, will help automate the assay and will lead to the method being applied routinely in clinical laboratories without the need for highly trained research personnel.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. In the examples all percentages are by weight, if for solids and by volume if for liquids, and all temperatures are in degrees Celsius unless otherwise noted.

EXAMPLE 1

The following conditions are currently in use to perform simultaneous amplification of a plurality of separate genomic regions within the human DMD gene. These conditions may need to be slightly modified depending on the particular regions to be amplified, the number and length of sequences to be amplified, and the choice of oligonucleotide primers. The time of reaction is highly dependent on the overall sequence length. Thus, as the number of amplified sequences increase and/or the length of amplified sequences increases, the time must be increased. The temperature is dependent on the length, the uniqueness of the primer sequence and the relative percentage of GC bases. The longer the primers, the higher the temperature needed. The more unique the sequence, the lower the temperature needed to amplify. GC rich primers need higher temperatures to prevent cross hybridization and to allow unique amplification. However, as the AT percentage increases, higher temperatures cause these primers to melt. Thus, these primers must be lengthened for the reaction to work.

Template DNA was prepared from the tissue chosen for analysis using a variety of well-established methods known to those skilled in the art. Typically, 100 µl reaction volumes were utilized. Approximately 500 ng of DNA was added to a solution comprised of the following: 67 mM Tris-HCL [pH 8.8 at 25°]; 6.7 mM magnesium chloride; 16.6 mM ammonium sulfate; 10 mM β-mercaptoethanol; 6.7 µM ethylene diamine tetra-acetic acid (EDTA); and 170 µg/mL bovine serum albumin. This solution can be prepared beforehand and appears to be stable for very long periods of storage at -70°. The enzyme, Tag polymerase, was added to achieve a final concentration of 100 units/mL. This reaction mixture was gently mixed. The reaction mixture was overlaid with about 50 µL of paraffin oil, and the reaction vessel (preferably a 0.5 ml microcentrifuge tube) was centrifuged at 14,000 x g for 10 sec. Amplification was performed either by manually transferring the reaction vessels between glycerol filled heat blocks at the appropriate temperatures, or automatically transferring the reaction vessels with a Perkin-Elmer/Cetus corporation thermocycler using the 'step-cycle' functions. The reaction was controlled by regulated and repetitive temperature changes of various duration. Initially the reaction was heated to 94° for 7 minutes. Subsequently 25 cycles of the following temperature durations were applied: 94° for 1 minute, then 55° for 45 seconds, then 65° for 3 1/2 minutes. Following completion of the final cycle the reaction was incubated at 65° for an additional 7 minutes. Reactions were then stored at 4° until analysis.

Genomic DNA deletions and/or exogenous DNA sequences were determined by examining the amplification products. For example, the lack of an expected amplification product indicates a deletion. Many methods for this determination are known to those skilled in the art. The preferred method involves electrophoresis of about one-twentieth of the reaction on a 1.4% (weight/vol) agarose gel in the following buffer: 40 mM tris-HCl; 20 mM sodium acetate, 1 mM EDTA (adjusted to pH 7.2 with glacial acetic acid), and 0.5µg/µl. of ethidium bromide. Electrophoresis was performed at 3.7 volts/cM for 100 minutes per 14 cM of agarose gel length. Analysis was completed by examining the electrophoresed reaction products on an ultraviolet radiation transilluminator, and the results were photographed for permanent records.

When the analysis requires determination of DNA sequence polymorphisms or mutations within individual amplification products the agarose gel is transferred to an appropriate DNA binding medium such as nitrocellulose using well-established procedures, for example, Southern blotting. Individual DNA sequences within the amplified DNA fragments can be determined by a variety of techniques including allele-specific oligonucleotide hybridization. Alternatively, reaction products may be further analyzed prior to electrophoresis on agarose gel by competitive oligonucleotide primer amplification, using separate allele-specific primers for each amplified DNA sequence of the multiplex amplification reaction products.

A third method for determining DNA sequence differences within individual amplification products does

not require electrophoresis. In this method, aliquots of the multiplex amplification reaction are sequentially applied to an appropriate DNA binding membrane such as nitrocellulose, and then each aliquot is analyzed via hybridization with individual members of sets of allele-specific oligonucleotide (ASO) probes, each separate aliquot being hybridized with one member of a pair of ASO probes specific for one member of the multiply amplified DNA sequences.

EXAMPLE 2

Figure 1 is a schematic representation of the DMD locus. The relative location of the exons used in the DMD gene amplification examples are illustrated.

For detection of DMD, a variety of probes can be used either in individual PCR reactions or in combinations in multiplex PCR reactions. These probes are shown in Table 1.

Table 1

Summary of DMD gene multiplex amplification primer sets.

Exon and Size		Primer Sequence	Amplified	Deleted
a.	Exon 8 (182bp)	F-GTCCTTTACACACTTTACCTGTTGAG R-GGCCTCATTCTCATGTTCTAATTAG	360 bp	11.3%
b.	Exon 17 (178bp)	F-GACTTTCGATGTTGAGATTACTTTCCC R-AAGCTTGAGATGCTCTCACCTTTTCC	416 bp	9.4%
c.	Exon 19 (88bp)	F-TTCTACCACATCCCATTTTCTTCCA R-GATGGCAAAAGTGTTGAGAAAAAGTC	459 bp	10.3%
d.	4.1Kb Hind III (148bp)	F-CTTGATCCATATGCTTTTACCTGCA R-TCCATCACCCTTCAGAACCTGATCT	268 bp	4.0%
e.	0.5Kb Hind III (176bp)	F-AAACATGGAACATCCTTGTGGGGAC R-CATTCCTATTAGATCTGTCGCCCTAC	547 bp	8.4%
f.	1.2/3.8Kb Hind III (159bp)	F-TTGAATACATTGGTTAAATCCCAACATG R-CCTGAATAAAGTCTTCCTTACCACAC	506 bp	18.2%
g.	Exon 12 (151bp)	F-GATAGTGGGCTTTACTTACATCCTTC R-GAAAGCACGCAACATAAGATACACCT	337 bp	9.6%
			Total:	38%

In Table 1 each exon is designated a, b, c, d, e, f, or g and corresponds to the same letter in Fig. 1. When the exon number is known it is listed. If the exon number is not known, the size of the genomic Hind III fragment containing that exon is listed. Also shown is the size of the exon in base pairs (bp). The PCR primer sequences are shown in 5′-3′ orientation. The forward primer (F), hybridizes 5′ of the exon, and the reverse primer (R), hybridizes 3′ of the exon. The size of the amplified fragment obtained with each primer set is also shown.

The percentage of analyzed DMD patients that are deleted for each indicated exon is shown in column four. This total number is less than the sum of the individual exon deletion frequencies because many deletions encompass multiple exons.

In Table 2 are the exon and flanking intron sequences for Exon 17. The exon is from 227 to 402. The primer sequences used to amplify this sequence are 7 to 33 and 396 to 421.

TABLE 2

```
5'        10         20         30         40         50
TAAATTGACT TTCGATGTTG AGATTACTTT CCCTTGCTAT TTCAGTGAAC
       60         70         80         90        100
CAAACTTAAG TCAGATAAAA CAATTTTATT TGGCTTCAAT ATGGTGCTAT
      110        120        130        140        150
TTTGATCTGA AGGTCAATCT ACCAACAAGC AAGAACAGTT TCTCATTATT
      160        170        180        190        200
TTCCTTTGCC ACTCCAAGCA GTCTTTACTG AAGTCTTTCG AGCAATGTCT
      210        220        230        240        250
GACCTCTGTT TCAATACTTC TCACAGATTT CACAGGCTGT CACCACCACT
      260        270        280        290        300
CAGCCATCAC TAACACAGAC AACTGTAATG GAAACAGTAA CTACGGTGAC
      310        320        330        340        350
CACAAGGGAA CAGATCCTGG TAAAGCATGC TCAAGAGGAA CTTCCACCAC
      360        370        380        390        400
CACCTCCCCA AAAGAAGAGG CAGATTACTG TGGATTCTGA AATTAGGAAA
      410        420        430        440        450
AGGTGAGAGC ATCTCAAGCT TTTATCTGCA AATGAAGTGG AGAAAACTCA
      460        470        480        490        500
TTTACAGCAG TTTTGTTGGT GGTGTTTTCA CTTCAGCAAT ATTTCCAGAA

      510        520        530        540        550
TCCTCGGGTA CCTGTAATGT CAGTTAATGT AGTGAGAAAA ATTATGAAGT
      560        570        580        590        600
ACATTTTAAA ACTTTCACAA GAAATCACTA TCGCAACAGA AACTAAATGC
      610        620        630        640        650
TTAATGGAAA TGGTGTTTTC TGGGGTGAAA GAAGAAACTA TAGAAACTAT
      660        670        680        690        700
AGGTGATAAA CTACTGTGGT AGCATTTTAA TCCTAAAAGT TTCTTTCTTT
      710        720        730        740        750
CTTTTTTTTT TTTCTTCCTT ATAAAGGGCC TGCTTGTTGA GTCCCTAGTT
      760        770        780        790        800
TTGCATTAAA TGTCTTTTTT TTCCAGTAAC GGAAAGTGCA TTTTCATGAA
      810        820        830        840        850
GAAGTACACC TATAATAGAT GGGATCCATC CTGGTAGTTT ACGAGAACAT
      860        870        880        890        900
GATGTCTCAG TCTGCGCATC CTAAATCAGG AGTAATTACA GAACACATTT
      910        920        930        940        950
CCTGTTCTTT GATATTTATA AAGTCTTATC TTGAAGGTGT TAGAATTTTT
      960        970        980        990       1000
AACTGATCTT TTTGTGACTA TTCAGAATTA TGCATTTTAG ATAAGATTAG
     1010       1020       1030       1040
GTATTATGTA AATCAGTGGA TATATTAAAT GATGGCAATA A-3'
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In Table 3 is the exon and flanking intron sequences for Exon d of Table 1 [or, the exon located on a 4.1 kb Hind III fragment]. The exon is from 295 to 442. The primer sequences used to amplify this sequence are 269 to 293 and 512 to 536.

TABLE 3

```
5'        10         20         30         40         50
TGTCCAAAAT AGTTGACTTT CTTTCTTTAA TCAATAAATA TATTACTTTA
          60         70         80         90        100
AAGGGAAAAA TTGCAACCTT CCATTTAAAA TCAGCTTTAT ATTGAGTATT
         110        120        130        140        150
TTTTTAAAAT GTTGTGTGTA CATGCTAGGT GTGTATATTA ATTTTTATTT
         160        170        180        190        200
GTTACTTGAA ACTAAACTCT GCAAATGCAG GAAACTATCA GAGTGATATC
         210        220        230        240        250
TTTGTCAGTA TAACCAAAAA ATATACGCTA TATCTCTATA ATCTGTTTTA
         260        270        280        290        300
CATAATCCAT CTATTTTTCT TGATCCATAT GCTTTTACCT GCAGGCGATT
         310        320        330        340        350
TGACAGATCT GTTGAGAAAT GGCGGCGTTT TCATTATGAT ATAAAGATAT
         360        370        380        390        400
TTAATCAGTG GCTAACAGAA GCTGAACAGT TTCTCAGAAA GACACAAATT
         410        420        430        440        450
CCTGAGAATT GGGAACATGC TAAATACAAA TGGTATCTTA AGGTAAGTCT
         460        470        480        490        500
TTGATTTGTT TTTTCGAAAT TGTATTTATC TTCAGCACAT CTGGACTCTT

         510        520        530        540        550
TAACTTCTTA AAGATCAGGT TCTGAAGGGT GATGGAAATT ACTTTTGACT
         560        570        580
GTTGTTGTCA TCATTATATT ACTAGAAAGA AAA-3'
```

In Table 4 is the exon and flanking Intron sequences for Exon e Table 1 [0.5 Kb Hind III fragment exon]. The exon is from 396 to 571. The primer sequences used to amplify this sequence are 51 to 75 and 572 to 597.

TABLE 4

```
5'        10                  20                  30                  40                  50
ACCCAAATAC  TTTGTTCATG  TTTAAATTTT  ACAACATTTC  ATAGACTATT
          60                  70                  80                  90                 100
AAACATGGAA  CATCCTTGTG  GGGACAAGAA  ATCGAATTTG  CTCTTGAAAA
         110                 120                 130                 140                 150
GGTTTCCAAC  TAATTGATTT  GTAGGACATT  ATAACATCCT  CTAGCTGACA
         160                 170                 180                 190                 200
AGCTTACAAA  AATAAAAACT  GGAGCTAACC  GAGAGGGTGC  TTTTTTCCCT
         210                 220                 230                 240                 250
GACACATAAA  AGGTGTCTTT  CTGTCTTGTA  TCCTTTGGAT  ATGGGCATGT
         260                 270                 280                 290                 300
CAGTTTCATA  GGGAAATTTT  CACATGGAGC  TTTTGTATTT  CTTTCTTTGC
         310                 320                 330                 340                 350
CAGTACAACT  GCATGTGGTA  GCACACTGTT  TAATCTTTTC  TCAAATAAAA
         360                 370                 380                 390                 400
AGACATGGGG  CTTCATTTTT  GTTTTGCCTT  TTTGGTATCT  TACAGGAACT
         410                 420                 430                 440                 450
CCAGGATGGC  ATTGGGCAGC  GGCAAACTGT  TGTCAGAACA  TTGAATGCAA
         460                 470                 480                 490                 500
CTGGGGAAGA  AATAATTCAG  CAATCCTCAA  AAACAGATGC  CAGTATTCTA
         510                 520                 530                 540                 550
CAGGAAAAAT  TGGGAAGCCT  GAATCTGCGG  TGGCAGGAGG  TCTGCAAACA
         560                 570                 580                 590                 600
GCTGTCAGAC  AGAAAAAAGA  GGTAGGGCGA  CAGATCTAAT  AGGAATGAAA
         610                 620
ACATTTTAGC  AGACTTTTTA  AGCTT-3'
```

In Table 5 is the exon and flanking intron sequences for Exon f, Table 1 [overlaps the 1.2 Kb and 3.8 Kb Hind III fragments]. The exon is from 221 to 406. The primer sequences used to amplify this sequence are 26 to 53 and 516 to 541.

TABLE 5

```
5'        10                  20                  30                  40                  50
TTTTGTAGAC  GGTTAATGAA  TAATTTTGAA  TACATTGGTT  AAATCCCAAC
          60                  70                  80                  90                 100
ATGTAATATA  TGTAAATAAT  CAATATTATG  CTGCTAAAAT  AACACAAATC
```

```
          110         120         130         140         150
AGTAAGATTC  TGTAATATTT  CATGATAAAT  AACTTTTGAA  AATATATTTT
          160         170         180         190         200
TAAACATTTT  GCTTATGCCT  TGAGAATTAT  TTACCTTTTT  AAAATGTATT
          210         220         230         240         250
TTCCTTTCAG  GTTTCCAGAG  CTTTACCTGA  GAAACAAGGA  GAAATTGAAG
          260         270         280         290         300
CTCAAATAAA  AGACCTTGGG  CAGCTTGAAA  AAAAGCTTGA  AGACCTTGAA
          310         320         330         340         350
GAGCAGTTAA  ATCATCTGCT  GCTGTGGTTA  TCTCCTATTA  GGAATCAGTT
          360         370         380         390         400
GGAAATTTAT  AACCAACCAA  ACCAAGAAGG  ACCATTTGAC  GTTAAGGTAG
          410         420         430         440         450
GGGAACTTTT  TGCTTTAATA  TTTTTGTCTT  TTTTAAGAAA  AATGGCAATA
          460         470         480         490         500
TCACTGAATT  TTCTCATTTG  GTATCATTAT  TAAAGACAAA  ATATTACTTG
          510         520         530         540         550
TTAAAGTGTG  GTAAGGAAGA  CTTTATTCAG  GATAACCACA  ATAGGCACAG
          560         570         580         590         600
GGACCACTGC  AATGGAGTAT  TACAGGAGGT  TGGATAGAGA  GAGATTGGGC
          610         620         630         640         650
TCAACTCTAA  ATACAGCACA  GTGGAAGTAG  GAATTTATAG  C-3'
```

In Table 6 is the exon and flanking intron sequences for Exon 12. The exon is from 180 to 329. The primer sequences used to amplify this sequence are 27 to 52 and 332 to 357.

TABLE 6

```
5'        10          20          30          40          50
TGAGAAATAA  TAGTTCCGGG  GTGACTGATA  GTGGGCTTTA  CTTACATCCT
          60          70          80          90         100
TCTCAATGTC  CAATAGATGC  CCCCAAATGC  GAACATTCCA  TATATTATAA
         110         120         130         140         150
ATTCTATTGT  TTTACATTGT  GATGTTCAGT  AATAAGTTGC  TTTCAAAGAG
         160         170         180         190         200
GTCATAATAG  GCTTCTTTCA  AATTTTCAGT  TTACATAGAG  TTTTAATGGA
         210         220         230         240         250
TCTCCAGAAT  CAGAAACTGA  AAGAGTTGAA  TGACTGGCTA  ACAAAACAGA
         260         270         280         290         300
AGAAAGAACA  AGGAAAATGG  AGGAAGAGCC  TCTTGGACCT  GATCTTGAAG
         310         320         330         340         350
ACCTAAAACG  CCAAGTACAA  CAACATAAGG  TAGGTGTATC  TTATGTTGCG
         360         370         380         390         400
TGCTTTCTAC  TAGAAAGCAA  ACTCTGTGTA  TAGTACCTAT  ACACAGTAAC
         410         420         430         440         450
ACAGATGACA  TGGTTGATGG  GAGAGAATTA  AAACTTAAAG  TCAGCCATAT
         460         470         480         490         500
TTTAAAAATT  ATTTTTACCT  AATTGTTTTT  GCAATCTTTG  TTGCCAATGG
         510         520         530         540         550
CCTTGAATAA  GTCCCCTCCA  AAATTCAGGT  GATTGTATTA  GGAGATGGAA
```

```
      560         570         580         590         600
TATTTAAGGG  TGAATAATCC  ATCAGGGCTC  CTCCCTTAAG  AATAGGATCA
      610         620         630         640         650
AGTCCCATAT  AAAAGAGGCT  TCACACAGTG  TTCTCCTATC  TCTTGACCCT
      660         670         680         690         700
CCACCATGCA  CCACCATGTG  AAAACTCTGT  GAAAAGGCCC  TCACCAGATG
      710         720         730         740         750
CTAACATCTT  GATCTTGGAT  TTCCCAAACT  CGAGAACTGT  GAAAAAATAA
      760         770         780         790         800
AGGTACATTC  TTCCTAAATT  ACCTCATTCT  CATTTAAACA  CACAAAGTGC
      810
ACACATAGCT  G-3'
```

In Table 7 is the exon and flanking intron sequences for the Exon located on a 10 Kb Hind III fragment. The exon is from 1 to 150.

TABLE 7

```
5'    10          20          30          40          50
TTACTGGTGG  AAGAGTTGCC  CCTGCGCCAG  GGAATTCTCA  AACAATTAAA
      60          70          80          90         100
TGAAACTGGA  GGACCCGTGC  TTGTAAGTGC  TCCCATAAGC  CCAGAAGAGC
     110         120         130         140         150
AAGATAAACT  TGAAAATAAG  CTCAAGCAGA  CAAATCTCCA  GTGGATAAAG
     160         170         180         190         200
GTTAGACATT  AACCATCTCT  TCCGTCACAT  GTGTTAAATG  TTGCAAGTAT
     210         220         230         240         250
TTGTATGTAT  TTTGTTTCCT  GGGTGCTTCA  TTGGTCGGGG  AGGAGGCTGG
     260         270         280
TATGTGGATT  GTTGTTTTGT  TTTGTTTTTT-3'
```

In Table 8 is the exon and flanking intron sequences for the Exon located on a 1.6 Kb Hind III fragment from 512 to 622.

TABLE 8

```
5'    10          20          30          40          50
AAGCTTTGAT  ACTGTGCTTT  AAGTGTTTAC  CCTTTGGAAA  GAAAATAATT
      60          70          80          90         100
TTGACAGTGA  TGTAGAAATA  ATTATTTGAT  ATTTATTTCA  AAACAAAATT
     110         120         130         140         150
TATATCCAAT  ACTAAACACA  GAATTTTGTA  AAACAATAAG  TGTATAAAGT
     160         170         180         190         200
AAAATGAACA  TTAGGATTAT  TGAGATTATT  GTAGCTAAAA  CTAGTGTTTA
     210         220         230         240         250
TTCATATAAA  TTATGTTAAT  AAATTGTATT  GTCATTATTG  CATTTTACTT
     260         270         280         290         300
TTTTGAAAAG  TAGTTAATGC  CTGTGTTTCT  ATATGAGTAT  TATATAATTC
```

310	320	330	340	350
AAGAAGATAT	TGGATGAATT	TTTTTTTTAA	GTTTAATGTG	TTTCACATCT
360	370	380	390	400
CTGTTTCTTT	TCTCTGCACC	AAAAGTCACA	TTTTTGTGCC	CTTATGTACC
410	420	430	440	450
AGGCAGAAAT	TGATCTGCAA	TACATGTGGA	GTCTCCAAGG	GTATATTTAA
460	470	480	490	500
ATTTAGTAAT	TTTATTGCTA	ACTGTGAAGT	TAATCTGCAC	TATATGGGTT
510	520	530	540	550
CTTTTCCCCA	GGAAACTGAA	ATAGCAGTTC	AAGCTAAACA	ACCGGATGTG
560	570	580	590	600
GAAGAGATTT	TGTCTAAAGG	GCAGCATTTG	TACAAGGAAA	AACCAGCCAC
610	620	630	640	650
TCAGCCAGTG	AAGGTAATGA	AGCAACCTCT	AGCAATATCC	ATTACCTCAT
660	670	680	690	700
AATGGGTTAT	GCTTCGCCTG	TTGTACATTT	GCCATTGACG	TGGACTATTT
710	720	730	740	750
ATAATCAGTG	AAATAACTTG	TAAGGAAATA	CTGGCCATAC	TGTAATAGCA
760	770	780	790	800
GAGGCAAAGC	TGTCTTTTTG	ATCAGCATAT	CCTATTTATA	TATTGTGATC
810	820	830	840	
TTAAGGCTAT	TAACGAGTCA	TTGCTTTAAA	GGACTCATTT	CTGTC-3'

In Table 9 is the exon and flanking intron sequences for the Exon located on a 3.1 Kb Hind III fragment. The exon is from 519 to 751.

TABLE 9

5' 103	113	123	133	143
CCCATCTTGT	TTTGCCTTTG	TTTTTTCTTG	AATAAAAAAA	AAATAAGTAA
153	163	173	183	193
AATTTATTTC	CCTGGCAAGG	TCTGAAAACT	TTTGTTTTCT	TTACCACTTC
203	213	223	233	243
CACAATGTAT	ATGATTGTTA	CTGAGAAGGC	TTATTTAACT	TAAGTTACTT
253	263	273	283	293
GTCCAGGCAT	GAGAATGAGC	AAAATCGTTT	TTTAAAAAAT	TGTTAAATGT
303	313	323	333	343
ATATTAATGA	AAAGGTTGAA	TCTTTTCATT	TTCTACCATG	TATTGCTAAA
353	363	373	383	393
CAAAGTATCC	ACATTGTTAG	AAAAAGATAT	ATAATGTCAT	GAATAAGAGT
403	413	423	433	443
TTGGCTCAAA	TTGTTACTCT	TCAATTAAAT	TTGACTTATT	GTTATTGAAA
453	463	473	483	493
TTGGCTCTTT	AGCTTGTGTT	TCTAATTTTT	CTTTTTCTTC	TTTTTTCCTT
503	513	523	533	543
TTTGCAAAAA	CCCAAAATAT	TTTAGCTCCT	ACTCAGACTG	TTACTCTGGT
553	563	573	583	593
GACACAACCT	GTGGTTACTA	AGGAAACTGC	CATCTCCAAA	CTAGAAATGC
603	613	623	633	643
CATCTTCCTT	GATGTTGGAG	GTACCTGCTC	TGGCAGATTT	CAACCGGGCT
653	663	673	683	693
TGGACAGAAC	TTACCGACTG	GCTTTCTCTG	CTTGATCAAG	TTATAAAATC

```
                703                 713                 723                 733                 743
ACAGAGGGTG  ATGGTGGGTG  ACCTTGAGGA  TATCAACGAG  ATGATCATCA
                753                 763                 773                 783                 793
AGCAGAAGGT  ATGAGAAAAA  ATGATAAAAG  TTGGCAGAAG  TTTTTCTTTA
                803                 813                 823                 833                 843
AAATGAAGAT  TTTCCACCAA  TCACTTTACT  CTCCTAGACC  ATTTCCCACC
                853                 863                 873                 883                 893
AGTTCTTAGG  CAACTGTTTC  TCTCTCAGCA  AACACATTAC  TCTCACTATT
                903                 913                 923                 933                 943
CAGCCTAAGT  ATAATCAGGT  ATAAATTAAT  GCAAATAACA  AAAGTAGCCA
                953                 963                 973                 983                 993
TACATTAAAA  AGGAAAATAT  ACAAAAAAAA  AAAAAAAAAA  AAGCCAGAAA
               1003                1013
CCTACAGAAT  AGTGCTCTAG  TAATTAC-3'
```

In Table 10 is the exon and flanking intron sequences for the Exon located on a 1.5 Kb Hind III fragment. The exon is from 190 to 337.

TABLE 10

```
5'        10                  20                  30                  40                  50
ATCTCTATCA  TTAGAGATCT  GAATATGAAA  TACTTGTCAA  AGTGAATGAA
                60                  70                  80                  90                 100
AATTTNNTAA  ATTATGTATG  GTTAACATCT  TTAAATTGCT  TATTTTTAAA
               110                 120                 130                 140                 150
TTGCCATGTT  TGTGTCCCAG  TTTGCATTAA  CAAATAGTTT  GAGAACTATG
               160                 170                 180                 190                 200
TTGGAAAAAA  AAATAACAAT  TTTATTCTTC  TTTCTCCAGG  CTAGAAGAAC
               210                 220                 230                 240                 250
AAAAGAATAT  CTTGTCAGAA  TTTCAAAGAG  ATTTAAATGA  ATTTGTTTTA
               260                 270                 280                 290                 300
TGGTTGGAGG  AAGCAGATAA  CATTGCTAGT  ATCCCACTTG  AACCTGGAAA
               310                 320                 330                 340                 350
AGAGCAGCAA  CTAAAAGAAA  AGCTTGAGCA  AGTCAAGGTA  ATTTTATTTT
               360                 370                 380                 390                 400
CTCAAATCCC  CCAGGGCCTG  CTTGCATAAA  GAAGTATATG  AATCTATTTT
               410                 420                 430                 440                 450
TTAATTCAAT  CATTGGTTTT  CTGCCCATTA  GGTTATTCAT  AGTTCCTTGC
               460                 470                 480                 490                 500
TAAAGTGTTT  TTCTCACAAC  TTTATTTCTT  CTTAACCCTG  CAGTTCTGAA
               510                 520                 530                 540                 550
CCAGTGCACA  TAAGAACATA  TGTATATATG  TGTGTGTGTG  TATTTATATA
               560                 570                 580                 590                 600
TACACACACA  CATATTGCAT  CTATACATCT  ACACATATAG  ATGTATAGAT
               610                 620                 630                 640                 650
TCAATATGTC  TAAAAATGTA  TATAATTCAC  AGTTTTTATC  TTTGATTTGA
               660                 670                 680
ATATTTAAGG  GACTGAGACT  CACACTCATA  TACTTTT-3'
```

EXAMPLE 3

Prenatal Diagnosis and Detection of DMD Using PCR

An example of prenatal diagnosis with PCR deletion detection is demonstrated using synthesized oligonucleotide primers (set b, Table 1). This primer set corresponds to the intron sequences flanking exon 17 of the human DMD gene, a region which has been isolated and sequenced (Table 2).

The results of this analysis are shown in figure 3. The PCR products (one-twentieth of the total reaction) were obtained with template DNA isolated from a control male □, the male fetus being diagnosed Λ, the DMD carrier mother (O) and an affected male brother of the fetus ■. Also shown is a DNA molecular weight standard (MW; Hae III digested ϕX174 DNA). The results demonstrate that the affected male carries a deletion of exon 17, which was not amplified, but that the fetus does not carry the deletion and is therefore unaffected. These results indicate that PCR is useful in the diagnosis of DMD cases containing a deletion involving this exon.

EXAMPLE 4

Multiplex Detection

An example of multiplex detection is shown in Figures 3A and 3B.

This analysis was done using six primer pairs (sets a-f, Table 1) and the conditions described in Example 1. Automatic rather than manual amplification was performed. These oligonucleotide primers represent the flanking regions of six separate DMD gene exons. They were combined into a reaction vial and used for multiplex genomic DNA amplifications. Template DNA was isolated from lymphoblasts (from blood sample). Analysis was by agarose gel electrophoresis.

When non-deleted DNA was used as a template, the six dispersed regions of the DMD gene were simultaneously and specificially amplified (Figure 3A, Sample #534). Discrete deletions, which were detected with this method, are shown in Figures 3A and 3B. Several DNA samples containing normal, partial or total DMD gene deletions are shown. Figures 3A and 3B also show a DNA molecular weight standard (MW: Hae III digested ϕX174 DNA), and a negative control (-) where no template DNA was added to the reactions. Figure 3A also indicates which amplified DNA fragment corresponds to which exon (a-f) of Figure 1.

EXAMPLE 5

Prenatal Diagnosis

Multiplex PCR has been used successfully in several prenatal diagnoses. The conditions are as described above in Example 1. Figure 4 shows Multiplex DNA amplification for prenatal diagnosis of DMD. Shown are the results of amplification using DNA from affected males (AM; lymphoblast DNA) and male fetuses (MF; cultured amniotic fluid cell DNA) from six different families. Analysis was as described in Example 1. Both the affected male and the fetal DNA of DRL #s 521 and 531 display a deletion of region f (Figure 1). Thus these fetuses were diagnosed as affected. In DRL # 43C the affected male is deleted for all regions except f, while the fetus is unaffected. The affected male in DRL #483 is deleted for region a, while the male fetus is unaffected. Neither of the samples from DRL #s 485 or 469 displayed a deletion with this technique. Thus, if a deletion defect causes DMD in these families it occurred in an untested exon.

EXAMPLE 6

Prenatal diagnosis using multiplex DNA amplification of chorionic villus specimen (CVS) DNA

Figure 5 demonstrates Multiplex DNA amplification from CVS DNA. Both the affected male (AM; lymphoblast DNA) and the male fetus (MF; CVS DNA) from DRL # 92 display a deletion of regions e and f (Fig. 1). Thus the fetus was diagnosed as affected. CVS DNA from DRL # 120 did not display a deletion with this technique. Samples were analyzed as described in Example 1. These results demonstrate that the multiplex amplification technique works well for prenatal diagnosis when CVS DNA is used as the template for amplification.

EXAMPLE 7

Multiplex amplification of seven separate exons of the DMD gene

This example demonstrates that seven separate DNA sequences can be simultaneously amplified using the multiplex amplification technique. Conditions were as described in Example 1. Primer sets a-g (Table 1) were added to the reaction. Thus seven exon regions of the DMD gene (Figure 1) were amplified (Figure 6).

EXAMPLE 8

Multiplex DNA amplification for the simultaneous detection of mutations leading to multiple common genetic diseases

This example describes how the multiplex amplification technique can be used to simultaneously screen a newborn male for any of the most common mutations leading to DMD, sickle-cell anemia and α_1-antitrypsin deficiency. In this assay any or all of the primers sets listed in Table 1 can be used for multiplex DNA amplification to diagnose the majority of possible DMD gene deletions. Additionally, primer sets can be added to the amplification reaction to identify mutations leading to additional genetic diseases. Other primer sets include:

A.

5′-TGGTCTCCTTAAACCTGTCTT-3′

5′-ACACAACTGTGTTCACTAG-3′

These oligonucleotides amplify a 167 bp segment of the human β-globin gene, containing the DNA base that is mutated in β^s (sickle-cell) hemoglobinopathy. The presence or absence of the mutant β^s sequence is then determined either by separate dot blot or Southern blot hybridization of the multiplex amplification reaction with each of two labelled allele-specific oligonucleotide (ASO) probes specific for the normal or β^s sequence. The sequence of these two ASO probes is:

1) Normal: 5′-CTCCTGAGGAGA-3′

2) β^s: 5′-CTCCTGTGGAGA-3′

If dot blot hybridization is used, a separate application of DNA from the multiplex amplification reaction to a DNA membrane, such as nitrocellulose, is required for each probe that will be used in the hybridization. Hybridization of each labelled probe, whether the probes are complementary to individual alleles of a given gene or to separate genes, must be performed individually. Alternatively and preferably, two aliquots of the amplification reaction are separately electrophoresed on agarose gels and transferred to nitrocellulose or a similar membrane using Southern analysis. Each of the two Southern blots are then hybridized with one member of each labelled set of specific ASO primers. Thus each known mutant or normal allele of each DNA fragment amplified in the multiplex reaction can be determined.

In addition to the above described primer sets the following oligonucleotide primers can also be added to the amplification procedure:

B.

5′-ACGTGGAGTGACGATGCTCTTCCC-3′
5′-GTGGGATTCACCACTTTTCCC-3′
These primers produce a 450 bp DNA fragment containing the DNA base change that produces the Z allele of the α_1-antitrypsin gene and leads to α_1-antitrypsin deficiency. The Z allele and the normal M allele are distinguished from other alleles in the multiplex amplification reaction by hybridization with the ASO probes:
1) Normal (M)allele:5′-ATCGACGAGAAA-3′
2) Mutant (Z)allele:5′-ATCGACAAGAAA-3′

Hybridization analysis is performed in parallel with the β-globin probes as described above.
In addition, the oligonucleotides
C.
5′-GAAGTCAAGGACACCGAGGAA-3′
5′-AGCCCTCTGGCCAGTCCTAGTG-3′
can also be added to the multiplex reaction to produce a 340 bp DNA region of the α_1-antitrypsin gene that contains the DNA base change that produces the S allele and leads to α_1-antitrypsin deficiency. The S allele is distinguished from other alleles in the multiplex amplification as described above for the β^s and Z alleles by using the two ASO probes specific for the M and S allele:
Normal (M)allele 5′-ACCTGGAAAATG-3′
Mutant (S)allele 5′-ACCTGGTAAATG-3′
Using the primers described in Table 1 and in A, B and C of this example the common mutations leading to DMD, sickle cell anemia and α_1-antitrypsin deficiency can be simultaneously determined.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well, those inherent therein. The methods procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary, and are not intended as limitations on the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

Claims

1. A method for simultaneously detecting deletions at a plurality of DNA sequences, comprising the steps of:
treating said DNA to form single-stranded complementary strands;
adding a plurality of paired oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense-strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand;
annealing the plurality of primers to their complementary sequences;
simultaneously extending said plurality of annealed primers from each primer's 3′ terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separation from their complement, serving as templates for the synthesis of an extension product from the other primer of each pair;
separating said extension products from said templates to produce single-stranded molecules;
amplifying said single stranded molecules by repeating, at least once, said annealing, extending and separating steps; and
identifying said amplified extension products from each different sequence.

2. The method of Claim 1 for detecting deletions at a plurality of genomic DNA sequences, wherein said sequences are selected from the group of sequences on the X and Y chromosomes.

3. The method of Claim 2 for the detection of X-linked disease, wherein said genomic DNA sequences contain a deletion that causes a genetic disease.

4. The method of Claim 3 for the detection of said X-linked genetic diseases selected from the group consisting of ornithine transcarbamylase deficiency, hypoxanthine phosphoribosyltransferase deficiency, steroid sulfatase deficiency and X-linked muscular dystrophy.

5. The method of Claim 4 for the detection of X-linked muscular dystrophy, wherein said plurality of paired primers are complementary to different sequences within the gene coding for the dystrophin protein.

6. The method of Claim 5, wherein the plurality of paired primers is selected from the group consisting of:

(1) 5′-GACTTTCGATGTTGAGATTACTTTCCC-3′
(2) 5′-AAGCTTGAGATGCTCTCACCTTTTCC-3′,
(1) 5′-GTCCTTTACACACTTTACCTGTTGAG-3′
(2) 5′-GGCCTCATTCTCATGTTCTAATTAG-3′,
(1) 5′-AAACATGGAACATCCTTGTGGGGAC-3′
(2) 5′-CATTCCTATTAGATCTGTCGCCCTAC-3′,
(1) 5′-GATAGTGGGCTTTACTTACATCCTTC-3′
(2) 5′-GAAAGCACGCAACATAAGATACACCT-3′,
(1) 5′-CTTGATCCATATGCTTTTACCTGCA-3′
(2) 5′-TCCATCACCCTTCAGAACCTGATCT-3′,
(1) 5′-GAATACATTGGTTAAATCCCAACATG-3′
(2) 5′-CCTGAATAAAGTCTTCCTTACCACAC-3′, and
(1) 5′-TTCTACCACATCCCATTTTCTTCCA-3′
(2) 5′-GATGGCAAAAGTGTTGAGAAAAAGTC-3′.

7. The method of Claim 3, wherein said genomic DNA is from fetal tissue.

8. The method of Claim 1 for detecting deletions at a plurality of genomic DNA sequences, wherein the plurality of paired primers is selected from the group consisting of:

(1) 5′-GACTTTCGATGTTGAGATTACTTTCCC-3′
(2) 5′-AAGCTTGAGATGCTCTCACCTTTTCC-3′,
(1) 5′-GTCCTTTACACACTTTACCTGTTGAG-3′
(2) 5′-GGCCTCATTCTCATGTTCTAATTAG-3′,
(1) 5′-AAACATGGAACATCCTTGTGGGGAC-3′
(2) 5′-CATTCCTATTAGATCTGTCGCCCTAC-3′,
(1) 5′-GATAGTGGGCTTTACTTACATCCTTC-3′
(2) 5 -GAAAGCACGCAACATAAGATACACCT-3′,
(1) 5′-CTTGATCCATATGCTTTTACCTGCA-3′
(2) 5′-TCCATCACCCTTCAGAACCTGATCT-3′,
(1) 5′-GAATACATTGGTTAAATCCCAACATG-3′
(2) 5′-CCTGAATAAAGTCTTCCTTACCACAC-3′,
(1) 5′-TTCTACCACATCCCATTTTCTTCCA-3′
(2) 5′-GATGGCAAAAGTGTTGAGAAAAAGTC-3′,
(1) 5′-TGGTCTCCTTAAACCTGTCTT-3′
(2) 5′-ACACAACTGTGTTCACTAG-3′,
(1) 5′-ACGTGGAGTGACGATGCTCTTCCC-3′
(2) 5′-GTGGGATTCACCACTTTTCCC-3′, and
(1) 5′-GAAGTCAAGGACACCGAGGAA-3′
(2) 5′-AGCCCTCTGGCCAGTCCTAGTG-3′.

9. A DNA sequence of the formula:

```
5'        10         20         30         40         50
TAAATTGACT TTCGATGTTG AGATTACTTT CCCTTGCTAT TTCAGTGAAC
          60         70         80         90        100
CAAACTTAAG TCAGATAAAA CAATTTTATT TGGCTTCAAT ATGGTGCTAT
         110        120        130        140        150
TTTGATCTGA AGGTCAATCT ACCAACAAGC AAGAACAGTT TCTCATTATT
         160        170        180        190        200
TTCCTTTGCC ACTCCAAGCA GTCTTTACTG AAGTCTTTCG AGCAATGTCT
         210        220        230        240        250
GACCTCTGTT TCAATACTTC TCACAGATTT CACAGGCTGT CACCACCACT
         260        270        280        290        300
CAGCCATCAC TAACACAGAC AACTGTAATG GAAACAGTAA CTACGGTGAC
```

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          310         320         330         340         350
CACAAGGGAA CAGATCCTGG TAAAGCATGC TCAAGAGGAA CTTCCACCAC
          360         370         380         390         400
CACCTCCCCA AAAGAAGAGG CAGATTACTG TGGATTCTGA AATTAGGAAA
          410         420         430         440         450
AGGTGAGAGC ATCTCAAGCT TTTATCTGCA AATGAAGTGG AGAAAACTCA
          460         470         480         490         500
TTTACAGCAG TTTTGTTGGT GGTGTTTTCA CTTCAGCAAT ATTTCCAGAA
          510         520         530         540         550
TCCTCGGGTA CCTGTAATGT CAGTTAATGT AGTGAGAAAA ATTATGAAGT
          560         570         580         590         600
ACATTTTAAA ACTTTCACAA GAAATCACTA TCGCAACAGA AACTAAATGC
          610         620         630         640         650
TTAATGGAAA TGGTGTTTTC TGGGGTGAAA GAAGAAACTA TAGAAACTAT
          660         670         680         690         700
AGGTGATAAA CTACTGTGGT AGCATTTTAA TCCTAAAAGT TTCTTTCTTT
          710         720         730         740         750
CTTTTTTTTT TTTCTTCCTT ATAAAGGGCC TGCTTGTTGA GTCCCTAGTT
          760         770         780         790         800
TTGCATTAAA TGTCTTTTTT TTCCAGTAAC GGAAAGTGCA TTTTCATGAA
          810         820         830         840         850
GAAGTACACC TATAATAGAT GGGATCCATC CTGGTAGTTT ACGAGAACAT
          860         870         880         890         900
GATGTCTCAG TCTGCGCATC CTAAATCAGG AGTAATTACA GAACACATTT
          910         920         930         940         950
CCTGTTCTTT GATATTTATA AAGTCTTATC TTGAAGGTGT TAGAATTTTT
          960         970         980         990        1000
AACTGATCTT TTTGTGACTA TTCAGAATTA TGCATTTTAG ATAAGATTAG
         1010        1020        1030        1040
GTATTATGTA AATCAGTGGA TATATTAAAT GATGGCAATA A-3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and antisense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

10. A DNA sequence of the formula:

```
5'        10          20          30          40          50
TGTCCAAAAT AGTTGACTTT CTTTCTTTAA TCAATAAATA TATTACTTTA
          60          70          80          90         100
AAGGGAAAAA TTGCAACCTT CCATTTAAAA TCAGCTTTAT ATTGAGTATT
         110         120         130         140         150
TTTTTAAAAT GTTGTGTGTA CATGCTAGGT GTGTATATTA ATTTTTATTT
         160         170         180         190         200
GTTACTTGAA ACTAAACTCT GCAAATGCAG GAAACTATCA GAGTGATATC
         210         220         230         240         250
TTTGTCAGTA TAACCAAAAA ATATACGCTA TATCTCTATA ATCTGTTTTA
         260         270         280         290         300
CATAATCCAT CTATTTTTCT TGATCCATAT GCTTTTACCT GCAGGCGATT
```

```
          310         320         330         340         350
TGACAGATCT  GTTGAGAAAT  GGCGGCGTTT  TCATTATGAT  ATAAAGATAT
          360         370         380         390         400
TTAATCAGTG  GCTAACAGAA  GCTGAACAGT  TTCTCAGAAA  GACACAAATT
          410         420         430         440         450
CCTGAGAATT  GGGAACATGC  TAAATACAAA  TGGTATCTTA  AGGTAAGTCT
          460         470         480         490         500
TTGATTTGTT  TTTTCGAAAT  TGTATTTATC  TTCAGCACAT  CTGGACTCTT
          510         520         530         540         550
TAACTTCTTA  AAGATCAGGT  TCTGAAGGGT  GATGGAAATT  ACTTTTGACT
          560         570         580
GTTGTTGTCA  TCATTATATT  ACTAGAAAGA  AAA-3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and antisense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

11. A DNA sequence of the formula:

```
5'        10          20          30          40          50
ACCCAAATAC  TTTGTTCATG  TTTAAATTTT  ACAACATTTC  ATAGACTATT
          60          70          80          90         100
AAACATGGAA  CATCCTTGTG  GGGACAAGAA  ATCGAATTTG  CTCTTGAAAA
         110         120         130         140         150
GGTTTCCAAC  TAATTGATTT  GTAGGACATT  ATAACATCCT  CTAGCTGACA
         160         170         180         190         200
AGCTTACAAA  AATAAAAACT  GGAGCTAACC  GAGAGGGTGC  TTTTTTCCCT
         210         220         230         240         250
GACACATAAA  AGGTGTCTTT  CTGTCTTGTA  TCCTTTGGAT  ATGGGCATGT
         260         270         280         290         300
CAGTTTCATA  GGGAAATTTT  CACATGGAGC  TTTTGTATTT  CTTTCTTTGC
         310         320         330         340         350
CAGTACAACT  GCATGTGGTA  GCACACTGTT  TAATCTTTTC  TCAAATAAAA
         360         370         380         390         400
AGACATGGGG  CTTCATTTTT  GTTTTGCCTT  TTTGGTATCT  TACAGGAACT
         410         420         430         440         450
CCAGGATGGC  ATTGGGCAGC  GGCAAACTGT  TGTCAGAACA  TTGAATGCAA
         460         470         480         490         500
CTGGGGAAGA  AATAATTCAG  CAATCCTCAA  AAACAGATGC  CAGTATTCTA
         510         520         530         540         550
CAGGAAAAAT  TGGGAAGCCT  GAATCTGCGG  TGGCAGGAGG  TCTGCAAACA
         560         570         580         590         600
GCTGTCAGAC  AGAAAAAAGA  GGTAGGGCGA  CAGATCTAAT  AGGAATGAAA
         610         620
ACATTTTAGC  AGACTTTTTA  AGCTT-3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and antisense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

12. A DNA sequence of the formula:

```
5'        10                  20                  30                  40                  50
TTTTGTAGAC  GGTTAATGAA  TAATTTTGAA  TACATTGGTT  AAATCCCAAC
          60                  70                  80                  90                 100
ATGTAATATA  TGTAAATAAT  CAATATTATG  CTGCTAAAAT  AACACAAATC
         110                 120                 130                 140                 150
AGTAAGATTC  TGTAATATTT  CATGATAAAT  AACTTTTGAA  AATATATTTT
         160                 170                 180                 190                 200
TAAACATTTT  GCTTATGCCT  TGAGAATTAT  TTACCTTTTT  AAAATGTATT
         210                 220                 230                 240                 250
TTCCTTTCAG  GTTTCCAGAG  CTTTACCTGA  GAAACAAGGA  GAAATTGAAG
         260                 270                 280                 290                 300
CTCAAATAAA  AGACCTTGGG  CAGCTTGAAA  AAAAGCTTGA  AGACCTTGAA
         310                 320                 330                 340                 350
GAGCAGTTAA  ATCATCTGCT  GCTGTGGTTA  TCTCCTATTA  GGAATCAGTT
         360                 370                 380                 390                 400
GGAAATTTAT  AACCAACCAA  ACCAAGAAGG  ACCATTTGAC  GTTAAGGTAG
         410                 420                 430                 440                 450
GGGAACTTTT  TGCTTTAATA  TTTTTGTCTT  TTTTAAGAAA  AATGGCAATA
         460                 470                 480                 490                 500
TCACTGAATT  TTCTCATTTG  GTATCATTAT  TAAAGACAAA  ATATTACTTG
         510                 520                 530                 540                 550
TTAAAGTGTG  GTAAGGAAGA  CTTTATTCAG  GATAACCACA  ATAGGCACAG
         560                 570                 580                 590                 600
GGACCACTGC  AATGGAGTAT  TACAGGAGGT  TGGATAGAGA  GAGATTGGGC
         610                 620                 630                 640                 650
TCAACTCTAA  ATACAGCACA  GTGGAAGTAG  GAATTTATAG  C-3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and antisense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

13. A DNA sequence of the formula:

```
5'        10                  20                  30                  40                  50
TGAGAAATAA  TAGTTCCGGG  GTGACTGATA  GTGGGCTTTA  CTTACATCCT
          60                  70                  80                  90                 100
TCTCAATGTC  CAATAGATGC  CCCCAAATGC  GAACATTCCA  TATATTATAA
         110                 120                 130                 140                 150
ATTCTATTGT  TTTACATTGT  GATGTTCAGT  AATAAGTTGC  TTTCAAAGAG
         160                 170                 180                 190                 200
GTCATAATAG  GCTTCTTTCA  AATTTTCAGT  TTACATAGAG  TTTTAATGGA
         210                 220                 230                 240                 250
TCTCCAGAAT  CAGAAACTGA  AAGAGTTGAA  TGACTGGCTA  ACAAAACAGA
```

```
          260         270         280         290         300
AGAAAGAACA  AGGAAAATGG  AGGAAGAGCC  TCTTGGACCT  GATCTTGAAG
          310         320         330         340         350
ACCTAAAACG  CCAAGTACAA  CAACATAAGG  TAGGTGTATC  TTATGTTGCG
          360         370         380         390         400
TGCTTTCTAC  TAGAAAGCAA  ACTCTGTGTA  TAGTACCTAT  ACACAGTAAC
          410         420         430         440         450
ACAGATGACA  TGGTTGATGG  GAGAGAATTA  AAACTTAAAG  TCAGCCATAT
          460         470         480         490         500
TTTAAAAATT  ATTTTTACCT  AATTGTTTTT  GCAATCTTTG  TTGCCAATGG
          510         520         530         540         550
CCTTGAATAA  GTCCCCTCCA  AAATTCAGGT  GATTGTATTA  GGAGATGGAA
          560         570         580         590         600
TATTTAAGGG  TGAATAATCC  ATCAGGGCTC  CTCCCTTAAG  AATAGGATCA
          610         620         630         640         650
AGTCCCATAT  AAAAGAGGCT  TCACACAGTG  TTCTCCTATC  TCTTGACCCT
          660         670         680         690         700
CCACCATGCA  CCACCATGTG  AAAACTCTGT  GAAAAGGCCC  TCACCAGATG
          710         720         730         740         750
CTAACATCTT  GATCTTGGAT  TTCCCAAACT  CGAGAACTGT  GAAAAAATAA
          760         770         780         790         800
AGGTACATTC  TTCCTAAATT  ACCTCATTCT  CATTTAAACA  CACAAAGTGC
          810
ACACATAGCT  G-3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and antisense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

14. A DNA sequence of the formula:

```
5'        10          20          30          40          50
TTACTGGTGG  AAGAGTTGCC  CCTGCGCCAG  GGAATTCTCA  AACAATTAAA
          60          70          80          90         100
TGAAACTGGA  GGACCCGTGC  TTGTAAGTGC  TCCCATAAGC  CCAGAAGAGC
         110         120         130         140         150
AAGATAAACT  TGAAAATAAG  CTCAAGCAGA  CAAATCTCCA  GTGGATAAAG
         160         170         180         190         200
GTTAGACATT  AACCATCTCT  TCCGTCACAT  GTGTTAAATG  TTGCAAGTAT
         210         220         230         240         250
TTGTATGTAT  TTTGTTTCCT  GGGTGCTTCA  TTGGTCGGGG  AGGAGGCTGG
         260         270         280
TATGTGGATT  GTTGTTTTGT  TTTGTTTTTT-3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and antisense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

15. A DNA sequence of the formula:

```
5'        10         20         30         40         50
AAGCTTTGAT ACTGTGCTTT AAGTGTTTAC CCTTTGGAAA GAAAATAATT
          60         70         80         90        100
TTGACAGTGA TGTAGAAATA ATTATTTGAT ATTTATTTCA AAACAAAATT
         110        120        130        140        150
TATATCCAAT ACTAAACACA GAATTTTGTA AAACAATAAG TGTATAAAGT
         160        170        180        190        200
AAAATGAACA TTAGGATTAT TGAGATTATT GTAGCTAAAA CTAGTGTTTA
         210        220        230        240        250
TTCATATAAA TTATGTTAAT AAATTGTATT GTCATTATTG CATTTTACTT
         260        270        280        290        300
TTTTGAAAAG TAGTTAATGC CTGTGTTTCT ATATGAGTAT TATATAATTC
         310        320        330        340        350
AAGAAGATAT TGGATGAATT TTTTTTTTAA GTTTAATGTG TTTCACATCT
         360        370        380        390        400
CTGTTTCTTT TCTCTGCACC AAAAGTCACA TTTTTGTGCC CTTATGTACC
         410        420        430        440        450
AGGCAGAAAT TGATCTGCAA TACATGTGGA GTCTCCAAGG GTATATTTAA
         460        470        480        490        500
ATTTAGTAAT TTTATTGCTA ACTGTGAAGT TAATCTGCAC TATATGGGTT
         510        520        530        540        550
CTTTTCCCCA GGAAACTGAA ATAGCAGTTC AAGCTAAACA ACCGGATGTG
         560        570        580        590        600
GAAGAGATTT TGTCTAAAGG GCAGCATTTG TACAAGGAAA AACCAGCCAC
         610        620        630        640        650
TCAGCCAGTG AAGGTAATGA AGCAACCTCT AGCAATATCC ATTACCTCAT
         660        670        680        690        700
AATGGGTTAT GCTTCGCCTG TTGTACATTT GCCATTGACG TGGACTATTT
         710        720        730        740        750
ATAATCAGTG AAATAACTTG TAAGGAAATA CTGGCCATAC TGTAATAGCA
         760        770        780        790        800
GAGGCAAAGC TGTCTTTTTG ATCAGCATAT CCTATTTATA TATTGTGATC
         810        820        830        840
TTAAGGCTAT TAACGAGTCA TTGCTTTAAA GGACTCATTT CTGTC-3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and antisense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

16. A DNA sequence of the formula:

```
5'       103        113        123        133        143
CCCATCTTGT TTTGCCTTTG TTTTTTCTTG AATAAAAAAA AAATAAGTAA
         153        163        173        183        193
AATTTATTTC CCTGGCAAGG TCTGAAAACT TTTGTTTTCT TTACCACTTC
```

```
          203         213         223         233         243
CACAATGTAT  ATGATTGTTA  CTGAGAAGGC  TTATTTAACT  TAAGTTACTT
          253         263         273         283         293
GTCCAGGCAT  GAGAATGAGC  AAAATCGTTT  TTTAAAAAAT  TGTTAAATGT
          303         313         323         333         343
ATATTAATGA  AAAGGTTGAA  TCTTTTCATT  TTCTACCATG  TATTGCTAAA
          353         363         373         383         393
CAAAGTATCC  ACATTGTTAG  AAAAAGATAT  ATAATGTCAT  GAATAAGAGT
          403         413         423         433         443
TTGGCTCAAA  TTGTTACTCT  TCAATTAAAT  TTGACTTATT  GTTATTGAAA
          453         463         473         483         493
TTGGCTCTTT  AGCTTGTGTT  TCTAATTTTT  CTTTTTCTTC  TTTTTTCCTT
          503         513         523         533         543
TTTGCAAAAA  CCCAAAATAT  TTTAGCTCCT  ACTCAGACTG  TTACTCTGGT
          553         563         573         583         593
GACACAACCT  GTGGTTACTA  AGGAAACTGC  CATCTCCAAA  CTAGAAATGC
          603         613         623         633         643
CATCTTCCTT  GATGTTGGAG  GTACCTGCTC  TGGCAGATTT  CAACCGGGCT
          653         663         673         683         693
TGGACAGAAC  TTACCGACTG  GCTTTCTCTG  CTTGATCAAG  TTATAAAATC
          703         713         723         733         743
ACAGAGGGTG  ATGGTGGGTG  ACCTTGAGGA  TATCAACGAG  ATGATCATCA
          753         763         773         783         793
AGCAGAAGGT  ATGAGAAAAA  ATGATAAAAG  TTGGCAGAAG  TTTTTCTTTA
          803         813         823         833         843
AAATGAAGAT  TTTCCACCAA  TCACTTTACT  CTCCTAGACC  ATTTCCCACC
          853         863         873         883         893
AGTTCTTAGG  CAACTGTTTC  TCTCTCAGCA  AACACATTAC  TCTCACTATT
          903         913         923         933         943
CAGCCTAAGT  ATAATCAGGT  ATAAATTAAT  GCAAATAACA  AAAGTAGCCA
          953         963         973         983         993
TACATTAAAA  AGGAAAATAT  ACAAAAAAAA  AAAAAAAAAA  AAGCCAGAAA
         1003        1013
CCTACAGAAT  AGTGCTCTAG  TAATTAC 3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and antisense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

17. A DNA sequence of the formula:

```
5'        10          20          30          40          50
ATCTCTATCA  TTAGAGATCT  GAATATGAAA  TACTTGTCAA  AGTGAATGAA
          60          70          80          90         100
AATTTNNTAA  ATTATGTATG  GTTAACATCT  TTAAATTGCT  TATTTTTAAA
         110         120         130         140         150
TTGCCATGTT  TGTGTCCCAG  TTTGCATTAA  CAAATAGTTT  GAGAACTATG
         160         170         180         190         200
TTGGAAAAAA  AAATAACAAT  TTTATTCTTC  TTTCTCCAGG  CTAGAAGAAC
```

210	220	230	240	250
AAAAGAATAT	CTTGTCAGAA	TTTCAAAGAG	ATTTAAATGA	ATTTGTTTTA
260	270	280	290	300
TGGTTGGAGG	AAGCAGATAA	CATTGCTAGT	ATCCCACTTG	AACCTGGAAA
310	320	330	340	350
AGAGCAGCAA	CTAAAAGAAA	AGCTTGAGCA	AGTCAAGGTA	ATTTTATTTT
360	370	380	390	400
CTCAAATCCC	CCAGGGCCTG	CTTGCATAAA	GAAGTATATG	AATCTATTTT
410	420	430	440	450
TTAATTCAAT	CATTGGTTTT	CTGCCCATTA	GGTTATTCAT	AGTTCCTTGC
460	470	480	490	500
TAAAGTGTTT	TTCTCACAAC	TTTATTTCTT	CTTAACCCTG	CAGTTCTGAA
510	520	530	540	550
CCAGTGCACA	TAAGAACATA	TGTATATATG	TGTGTGTGTG	TATTTATATA
560	570	580	590	600
TACACACACA	CATATTGCAT	CTATACATCT	ACACATATAG	ATGTATAGAT
610	620	630	640	650
TCAATATGTC	TAAAAATGTA	TATAATTCAC	AGTTTTTATC	TTTGATTTGA
660	670	680		
ATATTTAAGG	GACTGAGACT	CACACTCATA	TACTTTT-3'	

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and antisense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

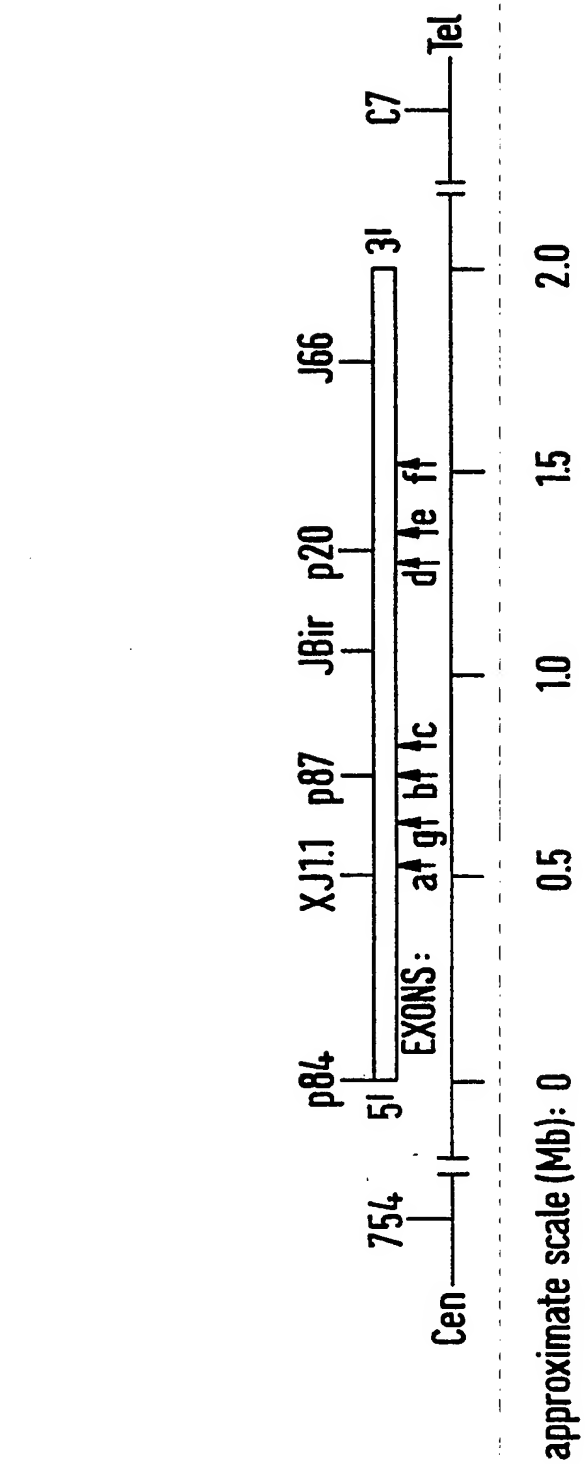
Cen
754
5'
p84
XJ1.1
p87
JBir
p20
J66
3'
C7
Tel
EXONS: a' g' b' c' d' e' f'
approximate scale (Mb): 0
0.5
1.0
1.5
2.0

FIG. 1

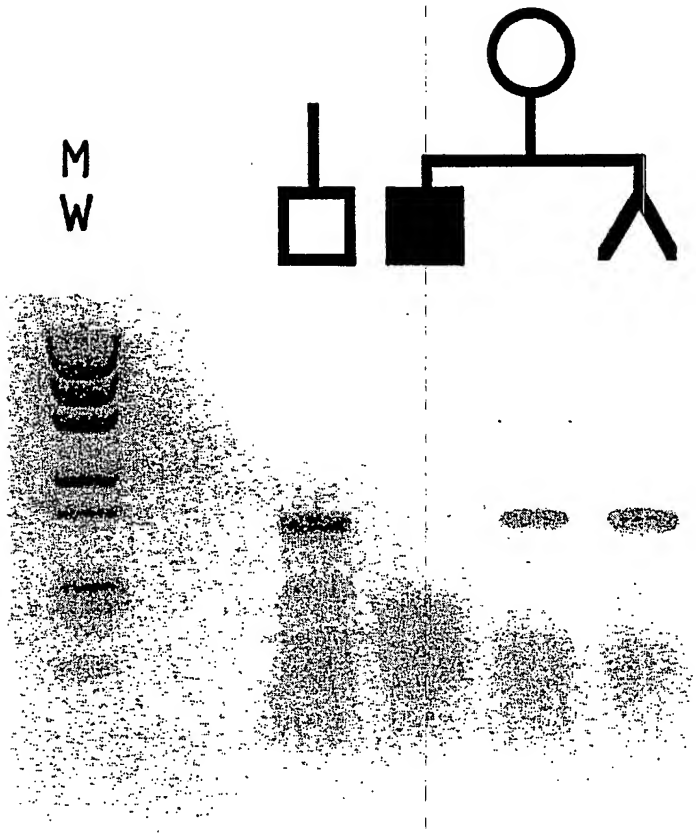

FIG.2

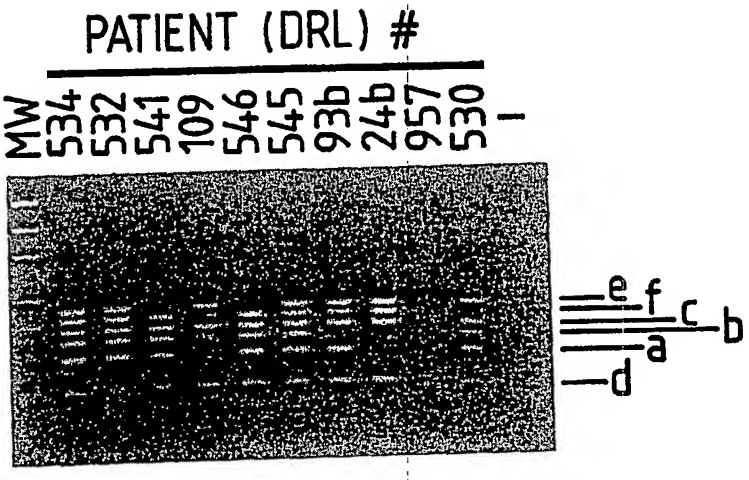
PATIENT (DRL) #
MW
534
532
541
109
546
545
93b
24b
957
530
—
e
f
c
b
a
d

FIG. 3A

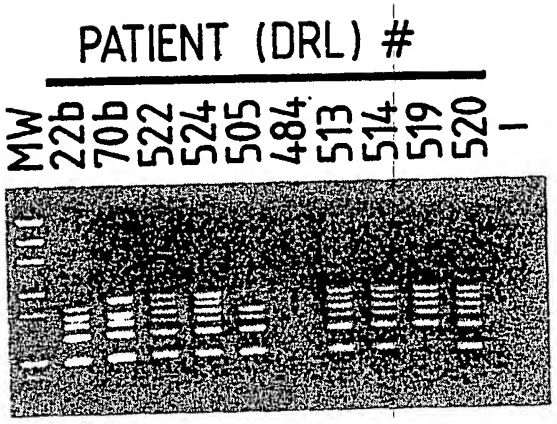
PATIENT (DRL) #
MW
22b
70b
522
524
505
484
513
514
519
520
—

FIG. 3B

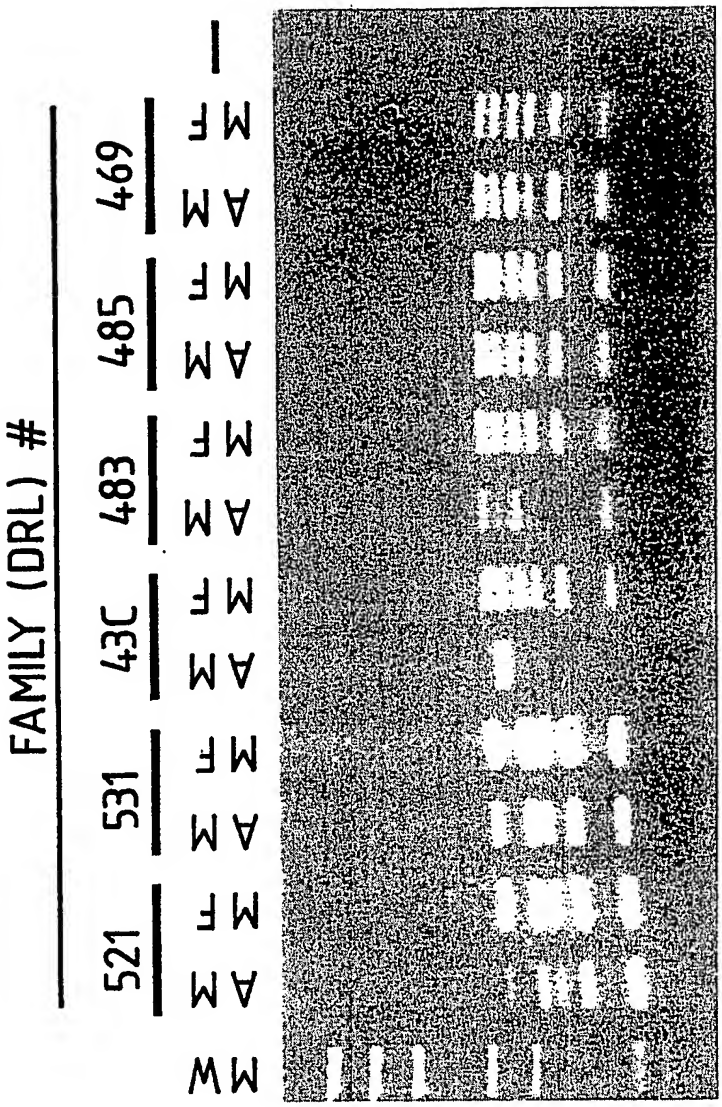

FIG.4

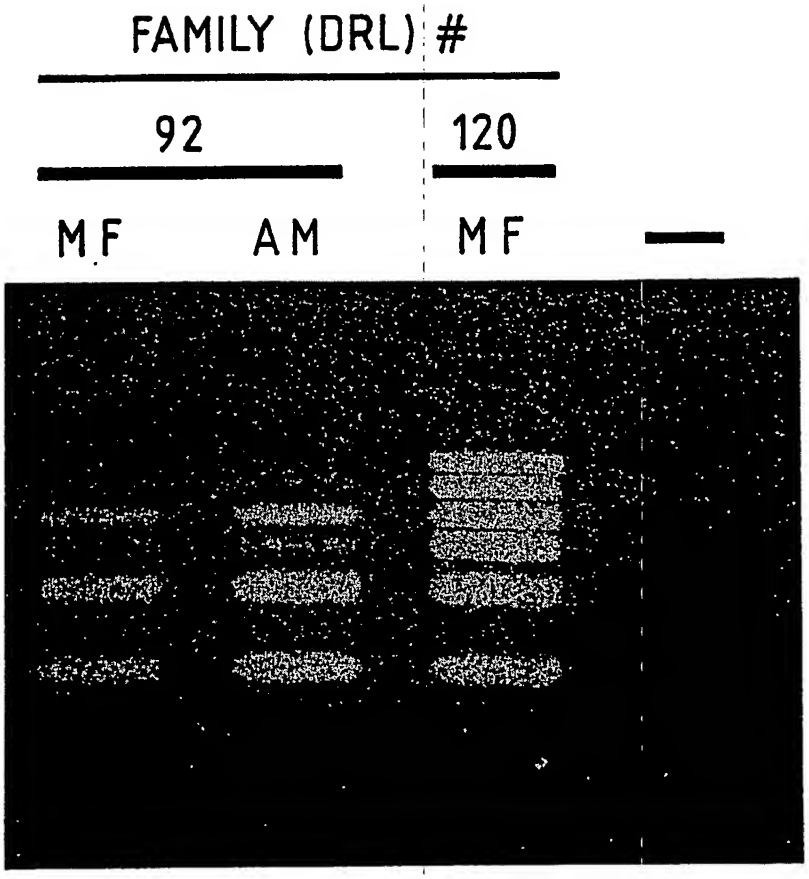

FIG.5

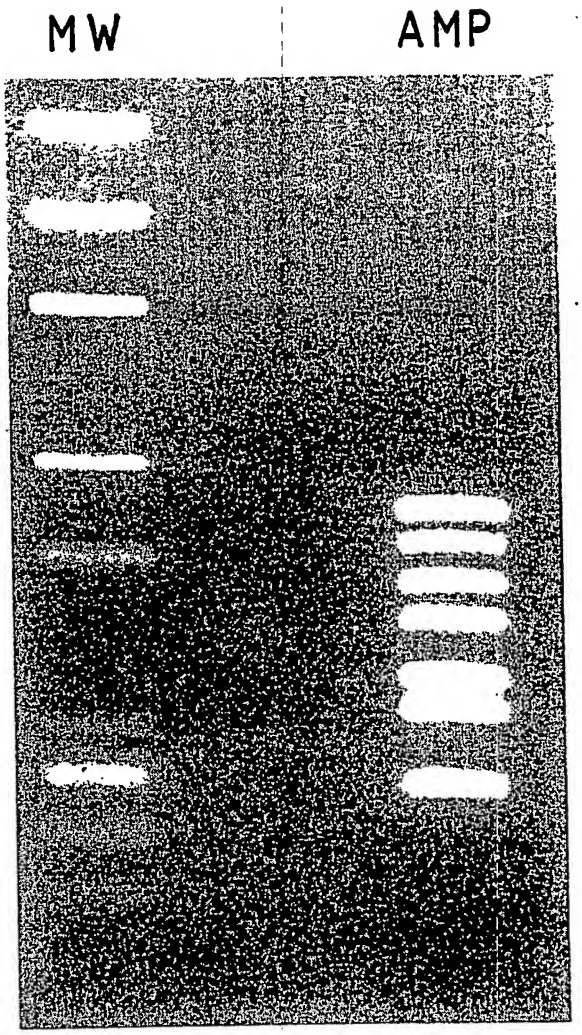

FIG.6